

# XANTHURENIC ACID AND ITS RÔLE IN THE TRYPTOPHANE METABOLISM OF PYRIDOXINE-DEFICIENT RATS\*

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In a previous publication (1) the isolation of a green pigment from the urine of pyridoxine-deficient rats was described. The green pigment was shown to be the product of a reaction between ferric ammonium sulfate or other ferric salts and a compound whose nature was unknown. This compound has now been isolated in crystalline form. It is a yellow pigment and has been identified as xanthurenic acid.

*Isolation of Xanthurenic Acid*—The xanthurenic acid was isolated from the urine of pyridoxine-deficient rats with the help of the paper-packed chromatographic column described in a previous publication (1). Cotton pulp has been used successfully and can replace the paper pulp. The yellow pigment was lightly adsorbed from rat urine<sup>1</sup> which had been saturated with sodium chloride. The column was washed with saturated sodium chloride solution, which carried through most of the urinary constituents before the yellow pigment came through. This process was repeated until the yellow pigment was obtained in a fair degree of purity. The sodium chloride-saturated solution containing the yellow pigment was then concentrated to about one-tenth of its volume and the precipitated sodium chloride removed by filtration. At this point, two different procedures were employed.

The solution was further concentrated until, along with sodium chloride, the yellow pigment crystallized out in needles which aggregated to form rosettes. The crystal form of the yellow pigment is illustrated in Fig. 1. The yellow pigment could be separated from the sodium chloride by recrystallization.

The second procedure consisted of eliminating most of the salt from the solution with alcohol and acetone. 2 to 4 volumes of alcohol were added to the salt-saturated solution and to this alcoholic solution was added an equal volume of acetone. The precipitate was discarded and the filtrate was

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<sup>1</sup> Much of the urine from pyridoxine-deficient rats was kindly given to us by the Vitab research laboratory, Emeryville, California.

evaporated in a vacuum until the yellow pigment crystallized out. It was recrystallized from solutions containing small amounts of sodium chloride. Attempts to recrystallize the yellow pigment from distilled water failed.

The yellow pigment differs in many ways from its green iron complex. The green pigment is more readily adsorbed on the paper-packed column than the yellow pigment, and so far it has not been obtained in the form of well defined crystals. The yellow pigment, like the green iron complex, is soluble in strong alcoholic solutions and in alcohol-acetone solutions. These solubility characteristics have been taken advantage of in the isolation of the yellow pigment, as described above.

*Identification of Yellow Pigment As Xanthurenic Acid*—The elementary analysis of the yellow pigment showed a considerable amount of ash, owing to the presence of sodium. To obtain the free acid, the pigment was dissolved in 50 per cent ethyl alcohol and the acid precipitated with 1 N

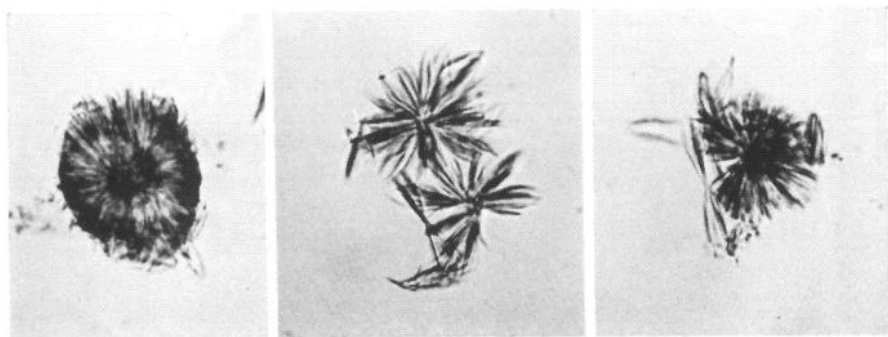


FIG. 1. Crystals of the Na salt of xanthurenic acid

$\text{H}_2\text{SO}_4$ . It was purified further by dissolving in warm 50 per cent alcohol, adding excess 0.1 N NaOH to form the sodium salt, and filtering off the impurities. The acid was reprecipitated with 0.1 N HCl. After two precipitations the melting point,  $288^\circ$ , of the substance could not be raised by further precipitation. The acid, which is a light yellow pigment, changes to yellowish brown in alkaline solution. When an excess of HCl is added, the free acid which is precipitated is redissolved, forming a light yellow-green solution. If the acid solution is left standing or is warmed, it turns nearly colorless. With methyl red it is monobasic; with phenolphthalein, dibasic.

The compound is nearly insoluble in water, slightly soluble in 50 per cent alcohol and in dilute HCl, and easily soluble in alkali hydroxides, carbonates, and hot dilute HCl.

The microanalysis gave the following results.

$\text{C}_{10}\text{H}_7\text{NO}_4$ .	Calculated.	C 58.53, H 3.42, N 6.83
	Found.	" 58.27, " 3.70, " 7.12

The equivalent weight was found by titration to be 103.3. Calculated for a dibasic acid of the formula  $C_{10}H_7NO_4$  = equivalent weight 102.5.

The analytical data are best explained by assuming that the yellow pigment is the sodium salt of a monohydroxyquinolinecarboxylic acid with 1 molecule of water of crystallization or that of a dihydroxyquinolinecarboxylic acid.

A search of the literature revealed that recently a similar acid had been found by Musajo (2) in urine. This acid is a 4,8-dihydroxyquinoline-2-carboxylic acid and was called xanthurenic acid. The properties of this xanthurenic acid resemble closely those of our yellow pigment.

Since it was not possible to obtain a sample of Musajo's acid and the melting points of kynurenic and xanthurenic acids are close together, and since other properties are also very similar, it was desirable to carry out specific color reactions and prepare the methyl ester for more reliable identification. The methyl ester of kynurenic acid melts at  $224^\circ$ , whereas the methyl ester of xanthurenic acid melts at  $262^\circ$ .

*Preparation of Methyl Ester of Xanthurenic Acid*—The free acid was suspended in absolute methyl alcohol (3) (25 mg. in 2 cc.). It was heated in a water bath at  $60^\circ$  and dry HCl bubbled through it for 3 hours. When the esterification was completed, the reaction mixture was cooled for 2 hours in ice-salt mixture, centrifuged, and washed twice with cold methyl alcohol. The precipitate was taken up in 25 cc. of boiling water and after being cooled in ice was neutralized with  $NaHCO_3$ . It was stirred again and allowed to stand overnight under refrigeration. The precipitate was recrystallized from hot 50 per cent ethyl alcohol. After three recrystallizations, 12.4 mg. of methyl ester were obtained with an approximate yield of 50 per cent. It crystallized as light yellow, silky needles which melted at  $260$ – $261^\circ$ . It was dried at  $110^\circ$  and analyzed.

$C_{11}H_9NO_4$ .	Calculated.	C 60.25, H 4.14, N 6.40
	Found.	" 60.32, " 4.25, " 6.52 <sup>2</sup>

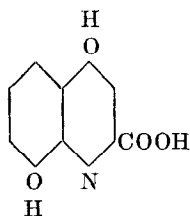
*The following color reactions were carried out with the free acid:* (1) Millon's reagent, orange-red color; (2) acetic acid-acetic anhydride and benzene, light brown-violet color; (3) freshly synthesized diazobenzosulfonic acid, intense red color.

In order to distinguish between the kynurenic acid and xanthurenic acid, reactions were carried out which gave different colors with these two quinolinecarboxylic acids (4). (1)  $FeCl_3$  added in the presence of  $NaHCO_3$  gave an intense green color. This is characteristic of xanthurenic acid, whereas kynurenic acid gives no color reaction. (2) Diazobenzosulfonic acid in the presence of  $NaHCO_3$  gave the red color of xanthurenic acid. Again kynu-

<sup>2</sup> The authors wish to express their thanks to Dr. G. Oppenheimer for carrying out the microanalyses.

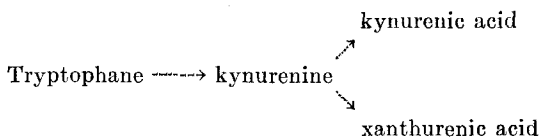
renic acid did not give this color reaction. (3) Xanthurenic acid evaporated with concentrated HCl and  $\text{KClO}_3$  turns brown when moistened with  $\text{NH}_3$ . Kynurenic acid similarly treated gave a green color. (4) The reaction of Kotake and Shichiri (5); the substance was boiled in alcoholic solution with phenylhydrazine hydrochloride and sodium carbonate and extracted, after being cooled, with ether. On addition of concentrated  $\text{H}_2\text{SO}_4$ , after removal of the ether, no color reaction was observed, whereas kynurenine gives a blue color.

The yellow compound isolated from the urine of pyridoxine-deficient rats must therefore be identical with xanthurenic acid, the formula of which is



*Relation of Pyridoxine Deficiency to Protein (Tryptophane) Metabolism—*

Since xanthurenic acid occurs in the urine of pyridoxine-deficient rats and immediately disappears from the urine on addition of pyridoxine to the diet, it would seem that xanthurenic acid is the result of some sort of metabolic derangement brought about in the rat by inadequate pyridoxine intake. That this metabolic derangement might involve tryptophane metabolism follows from the work of Musajo (6) who found that on high protein diets rats and rabbits excrete both kynurenic acid and xanthurenic acid in the urine. He suggested that these acids found their origin in tryptophane as follows:



If, in pyridoxine-deficient rats, xanthurenic acid originated from dietary tryptophane, the feeding of a tryptophane-deficient diet should result in the disappearance of xanthurenic acid from the urine of pyridoxine-deficient rats and the addition of tryptophane should cause the reappearance of xanthurenic acid in the urine of such rats. This has in fact been found experimentally to be the case.

Pyridoxine-deficient rats were put in metabolism cages<sup>3</sup> and the presence of xanthurenic acid in their urine was established by the production of the

<sup>3</sup> The authors wish to express their thanks to Mrs. Della Parsons for the care of the animals used in this work.

green color with ferric ammonium sulfate. The casein was then removed from their diets and replaced with zein and gelatin, a protein mixture very low in tryptophane. The xanthurenic acid disappeared from the rat urine in from 6 to 12 hours. Each rat was then fed 20 mg. daily of *l*-tryptophane and within 6 to 12 hours xanthurenic acid reappeared in their urine. Addition of 20  $\gamma$  of pyridoxine<sup>4</sup> daily to the diet of each rat immediately caused the disappearance of xanthurenic acid from the rat urine.

This experiment was repeated with acid-hydrolyzed casein replacing the casein in the pyridoxine-deficient diet. The absence of tryptophane in the acid-hydrolyzed casein was established by qualitative tests. The results obtained with this tryptophane-deficient diet were identical with those obtained with the use of zein and gelatin as the tryptophane-deficient proteins. This leaves little doubt that the function of pyridoxine is related to tryptophane metabolism.

These results are in harmony with those of Voris and Moore (7) who showed that pyridoxine is related to protein metabolism. They used paired rats, one rat of each pair receiving pyridoxine. The pyridoxine-fed rat gained more weight than its paired pyridoxine-deficient mate, and the gains in weight were exclusively protein, whereas in similar studies with thiamine and riboflavin the gains were represented by fat.

Foy and Cerecedo (8) studied pyridoxine deficiency in rats on diets containing 15, 30, and 45 per cent casein. At the low level of protein intake, little dermatitis developed in 70 days, while at the intermediate level the rats developed dermatitis after 30 days. At the highest level of protein intake (45 per cent casein) severe dermatitis developed in 26 days followed shortly after by death. Apparently high protein (tryptophane?) intake seems to increase the severity of the nutritional disorder in rats due to inadequate intake of pyridoxine. Perhaps the excess protein destroys pyridoxine in the rat in some unknown way. Such a phenomenon would explain the early appearance of xanthurenic acid in the urine of rats and rabbits (6) fed high protein diets.

*Species Differences*—While pyridoxine-deficient rats excrete an abundance of xanthurenic acid in their urines, pyridoxine-deficient dogs excrete very little (9), even when they have become very anemic as a result of the deficiency. So far, no xanthurenic acid has been demonstrated in the droppings of chicks.<sup>5</sup> Wintrobe *et al.* (10) have reported the occurrence of a compound in the urine of pyridoxine-deficient pigs which gives a green color with the addition of ferric ammonium sulfate, indicating the presence of xanthurenic acid.

<sup>4</sup> The pyridoxine was generously supplied by Merck and Company, Inc., Rahway, New Jersey.

<sup>5</sup> Kratzer, F. H., unpublished results.

## DISCUSSION

In the past, three related compounds, namely kynurenic acid, kynurenine, and xanthurenic acid, have been isolated from urine as products of protein metabolism. Kynurenic acid was first isolated by Liebig (11) in 1853, and Ellinger and Matsuoka (12) found it after feeding tryptophane. Kotake and Iwao (13) isolated kynurenine as an intermediary product in tryptophane metabolism and reported that kynurenine is excreted as a potassium salt, whereas kynurenic acid is found as a sodium salt. Musajo (2) found a compound similar to kynurenic acid in connection with feeding high protein diets and named it xanthurenic acid.

The excretion of kynurenic acid, kynurenine, and xanthurenic acid seems to be connected with vitamin deficiencies. Rabbits on a diet of polished rice, which is deficient in the vitamin B complex, excrete not only kynurenic acid but also kynurenine (13). In this paper it is shown that the urine of rats on a diet deficient in pyridoxine contains xanthurenic acid. Whether other members of the vitamin B complex are also related to tryptophane metabolism remains to be shown. Thus far all that is known is that pantothenic acid-deficient rats do not excrete any xanthurenic acid in their urine.

The species differences, especially as found with the dog and rat, are of interest. Pyridoxine-deficient rats excrete an abundance of xanthurenic acid in their urine, but they do not become so severely anemic as pyridoxine-deficient dogs (9); yet such dogs excrete but very little xanthurenic acid (9). It must follow, therefore, that the metabolism of tryptophane does not follow the same pathway in both the rat and the dog.

## SUMMARY

1. A yellow compound was isolated from the urine of pyridoxine-deficient rats.
2. This yellow compound is shown to be identical with Musajo's xanthurenic acid, a 4,8-dihydroxyquinoline-2-carboxylic acid.
3. Xanthurenic acid was shown to originate in dietary tryptophane.
4. The relation of pyridoxine to tryptophane and protein metabolism has been discussed.

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